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Heterochromatin loosening by the Oct4 linker facilitates Klf4 binding and iPSC reprogramming

Keshi Chen, Qi Long, Guangsuo Xing, Tianyu Wang, Yi Wu, Linpeng Li, Juntao Qi, Yanshuang Zhou, Bochao Ma, Hans R. Schöler, Jinfu Nie, Duanqing Pei, Xingguo Liu

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 7th Mar 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please accept my apologies for the extended duration of the review process of the manuscript at this time of the year. We have now received reports from three referees, which I copy below. In light of these comments, I am afraid we decided that we cannot offer publication in The EMBO Journal.

As you can see, the referees appreciate that the analysis extends previous work. However they also raise major concerns with the analysis that I am afraid preclude publication here. While referee #2 is overall more positive, referee #1 expresses major concerns regarding lack of novelty of the findings and in addition states that the claims on causalities between Oct4 linker mutation, Klf4 recruitment and histone mark alterations are not sufficiently well supported by the data, which in his/her view undermines the robustness of your work. Referee #3 agrees in that Oct4 and chromatin changes are not conclusively linked. In addition, the referees state major issues related to experimental documentation and controls missing and they request essential experiments to support and expand the concept made.

Given these negative opinions from good experts on the field, and that we need strong support from the referees to move on, I am afraid we cannot offer to publish your study in The EMBO Journal.

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REFEREE REPORTS:

Referee #1:

In this manuscript, the authors reported a role of the linker region of Oct4 on reprogramming. They found that the specific amino acid (L80) is required for the function of Oct4 as a pioneer factor to open condensed chromatin and mediate recruitment of Klf4. They also demonstrated that this function can be compensated by the enhancement of epigenetic reprogramming with vitamin C and Gadd45.

The functional importance of L80 was reported by Dr Schoeler's group previously (Esch et al, NCB, 2013) and it was suggested that L80 acts as a interface to recruit epigenetic regulators. Here the authors made an experimental proof of this hypothesis. The strategies were elegant and both interaction of Oct4 with the epigenetic factors and its functional significance in reprogramming were demonstrated. However, I wonder whether this paper provides sufficient novelty for publication in EMBO Journal. They showed (1) the interaction of Oct4 with Brg1 via the linker region, (2) increase of repressive histone marks (H3K9me3 and H3K27me3) in reprogramming with Oct4L80A, (3) decrease of Klf4 binding to the target sites in reprogramming with Oct4L80A, and (4) partial rescue of the reprogramming with Oct4L80A by Vit C or Gadd45. These events might be coupled with together, but there is no direct evidence. Brg1 is a component of BAF complex that mediates chromatin remodeling, and Vitamin C is known as an enhancer of Tet DNA demethylase. Gadd45 works as a heterochromatin relaxer that interrupts the interaction between core histones and double-stranded as the authors stated in the text. These functions have no direct link between them as well as with H3K9me3 and H3K27me3. Direct confirmation of functional sequence triggered by Oct4L80 will be required.

Referee #2:

In the manuscript (EMBOJ-2018-99165) by Chen et al., authors asked an important yet unanswered question in the field of basic research and regenerative medicine, namely during iPSC reprogramming, which Yamanaka factor(s) in somatic cells opens up the condensed chromatin state to its loose state, thus to facilitate the access of other factors to the target genes and initiate reprogramming? The authors adopted several techniques such as FRAP, Immunostaining and FISH to demonstrate that Oct4, but not Sox2 nor Klf4, is responsible for the loosening the condensed chromosome region. Oct4 is shown to execute its function via its linker domain with the aid of Brg1. Consequentially, this capability of Oct4 linker facilitates the binding of Klf4 and the expression of epithelial genes. Finally, the authors showed that this SKO-L80A-Oct4 linker mutation is an effective screening strategy for chromatin opener, by which they successfully screened out vitamin c and Gadd45a.
This work is both novel and important, and has satisfactorily answered, at least in part, a fundamental question as mentioned above. Particularly, the finding of a new role of Oct4 in heterochromatin remodeling and its cooperation with Klf4 in the initial phase represents a significant conceptual advance. This work also helps to widen our knowledge in that out of the four 'Yamanaka factors', Oct4 is the only one that is able to maintain a loose chromatin state in pluripotent stem cells. In general, the data and statistical analysis are solid enough to support their conclusions. The manuscript was well organized. In my opinion, this work is suitable for publication in EMBO Journal after some minor concerns to be properly addressed.

Minor concerns:
1, All over the manuscript, the authors used virus to infect cells. Please add the control of viral infection efficiency in supplementary figures.
2, The definition and calculation of mobile fraction of the FRAP should be given more details in the "Materials and methods" section.
3, Mesenchymal-to-epithelial transition (MET) is an important event in the early stage of reprogramming that benefits our understanding of reprogramming and helps to analyze the roles of reprogramming factors (PMID: 20621051, 23708003, 24805308, 25173869). Besides, more and more reports uncover the interplay between epigenetics and MET (PMID: 24529596, 25648270). The authors please emphasize this importance and add more recent advances about the the regulation of MET in reprogramming in Discussion section.
4, The different roles reprogramming factors play have been well studied (PMID: 19167336, 23159369, 23260147), so was the co-operation among them (PMID: 23747203, 20621050, 23708003). Oct4, Sox2 and Klf4 co-bind to the enhancers of genes that promote reprogramming. Klf4 organizes long-range chromosomal interactions leading to the activation of endogenous Oct4. In this study, the authors discovered a pioneer role of Oct4 in heterochromatin relaxation, which in turn enhances the Klf4's binding ability. Therefore, authors please add more specific information regarding the cooperations among these relevant factors during reprogramming to the Discussion part.

5, Typo: interrupt" in P9 should be "interrupts".
6. Quality of western blot shown in Fig.2c should be improved, especially the right part.
7. English language in this manuscript needs improvement before it is formally accepted.

Referee #3:

In this manuscript, the authors propose that Oct4 is required for chromatin decondensation during the early stages of mouse iPSC reprogramming. Using genetic and biochemical approaches they suggest that the linker region of Oct4 binds the chromatin remodeller Brg1, resulting in their recruitment to target sites and induction of active epigenetic marks and gene transcription. Mutating the linker region prevents reprogramming (as reported previously) but this defect can be rescued by treating cells with Vitamin C or Gadd45a.

The most significant insight is describing a new role for Oct4 in decondensing chromatin (uniquely amongst the reprogramming factors tested) and the description of a possible mechanism involving the direct interaction with the chromatin remodeller Brg1.

Although the topic is certainly interesting and some of the experiments have been well carried out, there are at least two major weaknesses: i) There is no evidence that Oct4 and chromatin remodelling are directly linked during reprogramming. It is equally plausible that the reported effects are indirect and that mutating the Oct4 linker domain hinders reprogramming through alternative processes, and that the failure of chromatin decondensation is a secondary effect. ii) There is insufficient evidence to support a specific role for Brg1 in this context, versus other chromatin remodellers and complexes. Given these weaknesses, my opinion is that the current manuscript is not supported by firm conclusions, and it lacks sufficient new and specific insight into the role of Oct4 in reprogramming.

Other major concerns include the absence of key experimental information for several figures, which makes it difficult to interpret the results. For example, Figures 1A and EV1 report FRAP, but of what protein(s)? HP1a and Histone H1? Please also show images of pre-bleach, bleach and recovering nuclei to see the targeted regions. It looks as though the experiments were only performed once. It is also difficult to get a sense of whether the SSEA-1-positive chromatin is 'hyderdynamic' without showing comparative data for MEF and iPSCs.

When comparing outcomes between different transcription factors, or between Oct4 and L80A Oct4, it is important to show that protein levels of the expressed factors are comparable.

Figure 1B. Are the levels of total HP1a lower in the Oct4 transduced cells compared to the other cells? That is a bit surprising given that HP1a is easily detectable in fully reprogrammed iPSCs.

Figure 1C. I can't really tell what I'm looking at here. And there is no attempt to quantify whether the Oct4 loci are inside or outside of chromocentres. Or replicates.

End of page 6: "These results indicated that the Oct4 loosens heterochromatin through its linker, by recruiting BAF complexes, which are known to regulate reprogramming". This conclusion is not supported by the results shown. The data provided shows that Oct4 and Brg1 co-localise (in CHO cells) and interact (when overexpressed in 293 cells), but does not say anything about the regulation of chromatin remodelling in the context of reprogramming or provide a direct role for Oct4 in heterochromatin control.

Figure 2C. Please add IgG control. The inputs, IgG control and IPs should be on same blot. The experiments should ideally be done in SSEA-1 positive reprogramming cells to show that endogenous proteins interact.

Figures 3 to 5 need MEF and iPSC samples as references in order to interpret the differences in ChIP levels.

1st Revision - authors' response 16th Jun 2019

[From the cover letter]

We are re-submitting our manuscript EMBOJ-2018-99165 titled "**Heterochromatin loosening by Oct4 linker facilitates Klf4's binding and initiates iPSC reprogramming**" for your further consideration as Article in *EMBO Journal*.

We are very happy to learn that referee #2 was overall more positive and referee #3 considered "the topic is certainly interesting", which encouraged us to improve the manuscript with more experiments. The reviewers also raised some comments, which are helpful for us to improve our manuscript. All comments were soluble, and we have performed experiments to answer these comments. Our manuscript has been improved much according to the reviewers' comments.

Referee #1:

In this manuscript, the authors reported a role of the linker region of Oct4 on reprogramming. They found that the specific amino acid (L80) is required for the function of Oct4 as a pioneer factor to open condensed chromatin and mediate recruitment of Klf4. They also demonstrated that this function can be compensated by the enhancement of epigenetic reprogramming with vitamin C and Gadd45.
The functional importance of L80 was reported by Dr Schoeler's group previously (Esch et al, NCB, 2013) and it was suggested that L80 acts as a interface to recruit epigenetic regulators. Here the authors made an experimental proof of this hypothesis. The strategies were elegant and both interaction of Oct4 with the epigenetic factors and its functional significance in reprogramming were demonstrated. However, I wonder whether this paper provides sufficient novelty for publication in EMBO Journal. They showed (1) the interaction of Oct4 with Brg1 via the linker region, (2) increase of repressive histone marks (H3K9me3 and H3K27me3) in reprogramming with Oct4L80A, (3) decrease of Klf4 binding to the target sites in reprogramming with Oct4L80A, and (4) partial rescue of the reprogramming with Oct4L80A by Vit C or Gadd45. These events might be coupled with together, but there is no direct evidence. Brg1 is a component of BAF complex that mediates chromatin remodeling, and Vitamin C is known as an enhancer of Tet DNA demethylase. Gadd45 works as a heterochromatin relaxer that interrupts the interaction between core histones and double-stranded as the authors stated in the text. These functions have no direct link between them as well as with H3K9me3 and H3K27me3. Direct confirmation of functional sequence triggered by Oct4L80 will be required.
Re: We do appreciate this comment, and have performed ATAC-seq to link the findings together, and Gadd45a wild-type/G39A rescuing SKO-L80A's the reprogramming deficiency and added new data and added new data (Fig 1C-I, 3A-G, 4A-F, 6A-H and EV2, EV3, EV4B-G).
First, we showed that Oct4 interacts with Brg1 directly in reprogramming depending on its L80 residue. Then, we designed shRNA of Brg1 to knockdown Brg1 in reprogramming and performed ATAC-seq. The normalized signal intensity of SKO with Brg1 silencing was lower than that of SKO, but higher than that of SKO-L80A. Most of the gene loci that failed to open (FO) in shBrg1 (90 out of 108) overlapped with FO in Oct4-L80A mutant. The states of specific gene loci such as Klf4 targets *Cdh1* and *Lefty*, epithelial gene *Ocln*, pluripotency genes *Oct4*, *Sox2* and *Nanog* were similar in SKO-L80A and SKO plus shBrg1. GO and motif analyses further indicated that knocking down Brg1 could partially mimic the effect of Oct4-L80A on reprogramming (Fig 4A-F and EV4B and C)
Second, we found that the ATAC-seq signal intensity at the endogenous *Oct4*, *Sox2* and *Nanog* loci were much lower in SKO-L80A than in SKO, consistent with the higher methylation levels of H3K9 and H3K27 in the promoter regions of these pluripotency genes in MEFs. Thus, the increased H3K9 and H3K27 methylation levels in SKO-L80A compared with SKO is also consistent with heterochromatin loosening.(Fig 3G-I)
Third, we discovered the decrease of Klf4 binding to the target sites in reprogramming with Oct4L80A and verified this finding using ATAC-seq. We analyzed the differences of open chromatin landscapes between SKO and SKO-L80A. GO analysis indicated these genes are involved in the transforming growth factor beta (TGF-β) signaling pathway, consistent with the retarded MET by Oct4-L80A mutation in reprogramming. Then we compared the differences in transcription factor motifs between SKO and SKO-L80A, and found that the gene loci in SKO-

L80A did not contain the Oct4:Sox2, Nanog, and Klf motifs. Moreover, the deficiency of Klf4's binding could also be seen at the specific loci; the target genes of Klf4 such as *Cdh1*, *Gata6*, *Tert*, *Mixl* and *Lefty*, and the epithelial genes such as *Ep-CAM* and *Ocln* failed to open in SKO-L80A. These results demonstrate that Oct4-L80A reduces the binding of Klf4 by affecting the heterochromatin loosening and therefore fails to activate the expression of epithelial genes.(Fig 3A-F and EV3)

Fourth, we showed partial rescue of the reprogramming with Oct4L80A by Vc or Gadd45. We also employed ATAC-seq to interrogate how Vc and Gadd45a affect the chromatin status in SKO-L80A reprogramming. Surprisingly, the normalized ATAC-seq signal intensity of SKO group showed that there were only 52 gene loci re-opened (RO) by Vc treatment in SKO-L80A reprogramming. Interestingly, most of these RO gene loci (50 out of 52 in Vc treatment) were overlapped with accessible gene loci in SKO compared with SKO-L80A. There were 445 RO gene loci in the presence of Gadd45a in SKO-L80A reprogramming, and similarly, most RO gene loci (407 out of 445) were overlapped with accessible gene loci in SKO compared with SKO-L80A. GO analysis showed that RO genes with Vc were involved in cell-cell adhesion while hormone metabolic processes were over-represented among Gadd45a RO genes, both of which categories are also enriched among the accessible genes in SKO. We identified several specific loci of the RO genes, such as *Echdc2*, re-opened by both Vc and Gadd45a, *Itsn2*, re-opened by Vc only, and *Ccdc42*, re-opened by Gadd45a only. We also found that both Vc and Gadd45a could re-open the pluripotency gene loci such as endogenous *Oct4*, *Sox2* and *Nanog*, and could reduce the methylation levels of H3K9 and H3K27 in the promoter regions of these pluripotency genes in SKO-L80A induced reprogramming. Moreover, the Klf motif was among the accessible gene loci in both Vc treatment and Gadd45a overexpression in SKO-L80A, suggesting they could rescue the binding of Klf4. Indeed, *Cdh1* and *Lefty* could be re-opened by both Vc and Gadd45a, while *Tert* and *Mixl* could be re-opened by Gadd45a. ChIP-PCR results indicated that both Vc and Gadd45a greatly enhanced the binding of Klf4 in SKO-L80A infected MEFs. Finally, *Ep-CAM* could be re-opened by Vc and *Ocln* could be re-opened by Gadd45a, and the expression of epithelial genes including *Cdh1*, *Ep-CAM* and *Ocln* increased with both Vc and Gadd45a in SKO-L80A induced reprogramming. The re-activation of Cdh1 by Vc and Gadd45a was further confirmed by western blot. Thus, our results demonstrated Vc or Gadd45a complements Oct4-L80A's heterochromatin loosening defects to rescue reprogramming efficiency. (Fig 6B-K and EV4E-H)

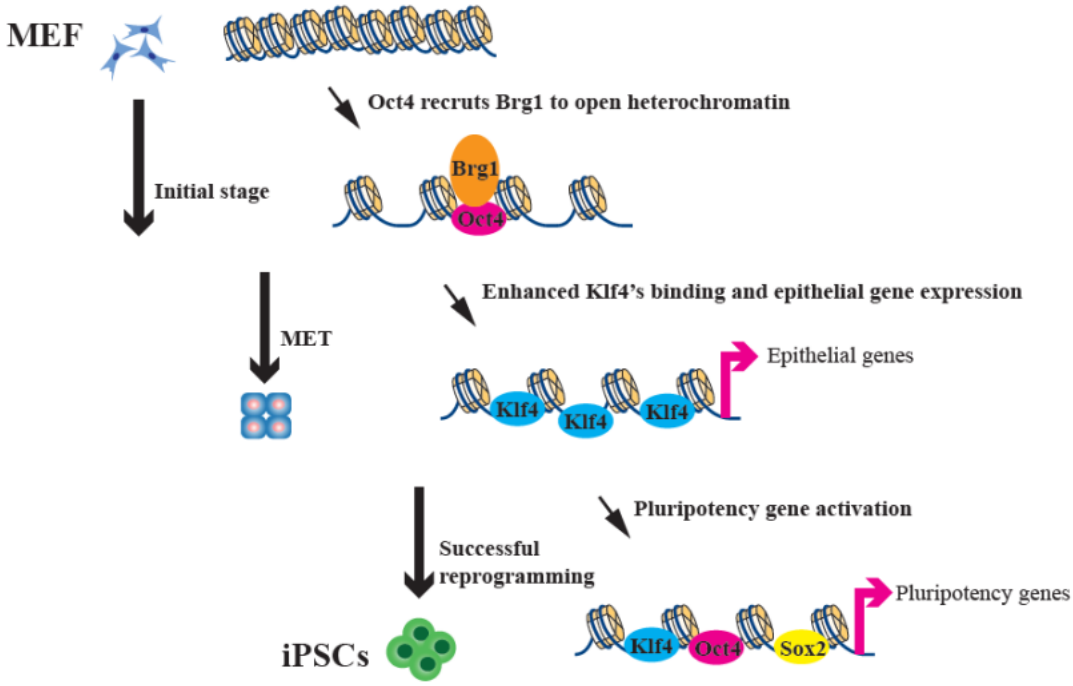
Fifth, as in previous report we indicated that Gadd45a opens up heterochromatin depending on its G39 residue (Chen et al., 2016), we have performed reprogramming rescue experiments and showed that Gadd45a could rescue the reprogramming deficiency of SKO-L80A, while its mutant Gadd45a-G39A couldn't (Fig 6A and EV4D). As Gadd45 is a heterochromatin loosener and G39A mutant loses this function, our rescue data strongly demonstrated Oct4 loosens heterochromatin through its linker, but little chance of some same specific transcriptional responses via both G39 of Gadd45 and L80 of Oct4. We have added text: "Gadd45a, as well as other family members Gadd45b and Gadd45g, but not its inactive form Gadd45a-G39A, were able to rescue SKO-L80A (Fig 6A and EV4D). As Gadd45a opens up heterochromatin depending on the G39 residue (Chen et al., 2016), our rescue data implicate that the reprogramming deficiency of SKO-L80A should be due to the failure of heterochromatin loosening by Oct4-L80A similar as Gadd45a-G39A."

Sixth, we have added text to demonstrate "the linkage between partial rescue of the reprogramming with Oct4L80A by Vit C or Gadd45" and "increase of repressive histone marks (H3K9me3 and H3K27me3) in reprogramming with Oct4L80A", We showed the overexpression of Gadd45a could reduce the H3K9Me3 and H3K27Me3 levels significantly in reprogramming in previous reports (Chen et al., 2016). Vc has wide impacts on epigenetic modifications in reprogramming such as H3K9Me3 demethylation and DNA demethylation (Chen et al., 2013a, Chen et al., 2013b). As the histone deacetylase inhibitor valproic acid (VPA), and DNA methyltransferase inhibitor 5'-azacytidine (5'-az-A) couldn't rescue SKO-L80A reprogramming, we ruled out that histone acetylation and DNA demethylation involved in Oct4 loosening heterochromatin in reprogramming. These results suggested that Vc rescue the reprogramming deficiency of SKO-L80A through H3K9Me3 demethylation. We have added text: "We then tried another reprogramming enhancer, Gadd45a, a heterochromatin relaxer that interrupts the interactions between core histones and double-stranded DNA, and could also reduce the H3K9Me3 and H3K27Me3 levels significantly in reprogramming (Carrier et al., 1999, Chen et al., 2016)."

"In our previous work, we have reported that Vc has wide impacts on epigenetic modifications in reprogramming such as H3K9Me3 demethylation and DNA demethylation (Chen et al., 2013a, Chen et al., 2013b). As VPA and 5'-az-A couldn't rescue SKO-L80A reprogramming, we ruled out that histone acetylation and DNA demethylation involved in Oct4 loosening heterochromatin in

reprogramming and suggested that Vc modulates H3K9me3 to open heterochromatin, facilitating the expression of endogenous Oct4 (Chen et al., 2013b), a possible mechanism for the rescue of SKO-L80A by Vc.''

Altogether, by ATAC-seq and other assays, we showed that in reprogramming heterochromatin is opened by Oct4, dependent on its L80 residue. L80A-Oct4 inhibits Klf4's binding and the expression of epithelial genes in early reprogramming. In late reprogramming, L80A Oct4 failed to activate pluripotency genes. In addition, vitamin C and Gadd45 proteins were identified as capable of restoring the reprogramming deficiency of SKO-L80A in both early and late reprogramming. The summary was showed in Fig 7 and the picture below.



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Minor concerns:

1, All over the manuscript, the authors used virus to infect cells. Please add the control of viral infection efficiency in supplementary figures.

Re: We do appreciate this comment, and have added the control of viral infection efficiency as suggested (Fig EV1A). As shown in Fig EV1A, the viral infection efficiency in our experiments was almost 100%.

2, The definition and calculation of mobile fraction of the FRAP should be given more details in the "Materials and methods" section.
Re: We do appreciate this comment, and have added the definition and calculation of mobile fraction of the FRAP both in “Materials and methods” and in “Fig EV1C legends”. In the "Materials and methods" section, we added “The curves are divided into two parts—the mobile fraction (MF) and the immobile fraction (IF) after bleaching recovery and the MF was used to be analyzed.” And also in Fig EV1C legend: “The relative FRAP curves of heterochromatin (Het) are shown divided into two parts—the mobile fraction (MF) and the immobile fraction (IF) after bleaching recovery.”

3, Mesenchymal-to-epithelial transition (MET) is an important event in the early stage of reprogramming that benefits our understanding of reprogramming and helps to analyze the roles of reprogramming factors (PMID: 20621051, 23708003, 24805308, 25173869). Besides, more and more reports uncover the interplay between epigenetics and MET (PMID: 24529596, 25648270). The authors please emphasize this importance and add more recent advances about the the regulation of MET in reprogramming in Discussion section.
Re: We do appreciate this comment, and have added it in Discussion section: “We and others have reported that mesenchymal-to-epithelial transition is an early event essential for MEFs to be successfully reprogrammed into iPSCs (Hu et al., 2014, Li et al., 2010, Samavarchi-Tehrani et al., 2010). Among the three reprogramming factors, Klf4 directly binds to the promoter of Cdh1, an important regulator of epithelial homeostasis, and activates an epithelial gene expression program including Ep-CAM and Ocln, while Oct4 inhibits the mesenchymal gene Snail by suppressing TGFb signaling (Li et al., 2010, Shu & Pei, 2014). We described a new role of Oct4 to activate the epithelial genes expression by enhancing the binding of Klf4. Our findings link heterochromatin relaxation induced by Oct4 and mesenchymal-to-epithelial transition induced by Klf4 together, and indicate that organized cooperation of the reprograming factors leads to successful reprogramming. Furthermore, our results suggest an early requirement for Oct4, rather than a late one, during the reprogramming process. Indeed, our previous work has reported the optimal sequential delivery of Oct4/Klf4, Myc, and Sox2 and a sequential EMT-MET mechanism for optimal reprogramming (Li et al., 2014, Liu et al., 2013).
MET and EMT play critical roles in embryonic development and are also strongly associated with tumors. Due to the direct binding and activation of Cdh1, a key epithelial marker gene, Klf4 participates in many biological processes through MET and EMT regulation (Evans & Liu, 2008). Klf4 is widely expressed in many tissues including intestine, eys, skin, bone and teeth, testis, vascular smooth muscle cells, blood cells and kidney and participates in the formation of epithelial permeability barrier (Ghaleb & Yang, 2017). Klf4 plays critical roles in self-renewal and pluripotency of mESCs. Overexpression of Klf4 in mESCs could inhibit their differentiation into both hemangioblasts and primitive erythroid progenitors while retaining a high capacity to generate secondary embryoid bodies (Li et al., 2005). Klf4, along with Klf2 and Klf5, forms a circuitry that promotes self-renewal by activating pluripotency genes and inhibiting their differentiation into endoderm and mesoderm (Jiang et al., 2008). There is evidence that Klf4 may assist Oct4 and Sox2 to stimulate key mESC genes such as Lefty in somatic cells (Nakatake et al., 2006), while, in our study, we showed Oct4 facilitates Klf4’s binding to Lefty. These results suggested close cooperation between Oct4 and Klf4 to activate the key pluripotency genes in pluripotency induction and maintenance.”

4, The different roles reprogramming factors play have been well studied (PMID: 19167336, 23159369, 23260147), so was the co-operation among them (PMID: 23747203, 20621050, 23708003). Oct4, Sox2 and Klf4 co-bind to the enhancers of genes that promote reprogramming. Klf4 organizes long-range chromosomal interactions leading to the activation of endogenous Oct4. In this study, the authors discovered a pioneer role of Oct4 in heterochromatin relaxation, which in turn enhances the Klf4's binding ability. Therefore, authors please add more specific information regarding the cooperation among these relevant factors during reprogramming to the Discussion part.
Re: We do appreciate this comment, and have added it in Discussion section: “We showed that Oct4 is the pioneer factor that opens up the heterochromatin through its linker and facilitates Klf4’s binding during the initial phase of SKO mediated reprogramming, which provides novel cooperation action of S, K and O in reprogramming (Fig 7). Consistent with us, Polo and colleagues showed that Oct4/Sox2 pioneers the opening of chromatin during SKOM mediated reprogramming (Polo et al., 2012). However, they indicated that Oct4/Sox2 binding is transient at the early stage and that Klf4

recruits Oct4/Sox2 to its target loci to loosen the closed chromatin (Knaupp et al., 2017). Other reports also showed Oct4/Sox2/Klf4 as pioneers and identified their specific binding sites and co-binding sites, and the role of c-Myc in facilitating OSK chromatin engagement (Soufi et al., 2012). OSK predominantly bind and close the somatic enhancers and then switch to bind and open the pluripotency enhances (Chronis et al., 2017). Zaret et al. analyzed the DNA binding domains of OSK, and found the bipartite POU domain of Oct4 could not only bind DNA but also interact with histones, while Klf4 prefers to bind free DNA, supporting our finding that Oct4 could affect Klf4's binding through chromatin loosening (Soufi et al., 2015)."

5, Typo: interrupt" in P9 should be "interrupts".
Re: We have corrected this typo.

6. Quality of western blot shown in Fig.2c should be improved, especially the right part.
Re: We do appreciate this comment, and have done more experiments. We have changed a more clearly image in the figure.

7. English language in this manuscript needs improvement before it is formally accepted.
Re: We do appreciate this comment, and have rewrote the manuscript to improve the language.

Referee #3:

In this manuscript, the authors propose that Oct4 is required for chromatin decondensation during the early stages of mouse iPSC reprogramming. Using genetic and biochemical approaches they suggest that the linker region of Oct4 binds the chromatin remodeller Brg1, resulting in their recruitment to target sites and induction of active epigenetic marks and gene transcription. Mutating the linker region prevents reprogramming (as reported previously) but this defect can be rescued by treating cells with Vitamin C or Gadd45a.

The most significant insight is describing a new role for Oct4 in decondensing chromatin (uniquely amongst the reprogramming factors tested) and the description of a possible mechanism involving the direct interaction with the chromatin remodeller Brg1.

Although the topic is certainly interesting and some of the experiments have been well carried out, there are at least two major weaknesses: i) There is no evidence that Oct4 and chromatin remodelling are directly linked during reprogramming. It is equally plausible that the reported effects are indirect and that mutating the Oct4 linker domain hinders reprogramming through alternative processes, and that the failure of chromatin decondensation is a secondary effect.
Re: We do appreciate this comment. Besides FRAP and HP1a staining, we have performed ATAC-seq, and Gadd45a wild-type/G39A rescuing SKO-L80A's the reprogramming deficiency and added new data (Fig 1C-I, 3A-G, 4A-F, 6A-H and EV2, EV3, EV4B-G).
First, among the three Yamanaka factors, although much lower than in SKO, the normalized ATAC-seq signal intensity and the accessible gene loci were most prominent in Oct4, which is consistent with our FRAP results that Oct4 is responsible for the heterochromatin loosening in early reprogramming. Then we compared the differences of chromatin landscape between Oct4 and Oct4-L80A. The normalized ATAC-seq signal intensity in Oct4-L80A was much lower than in Oct4, and even lower than in Flag control. There were few accessible gene loci in Oct4-L80A, compared with in Flag, and most gene loci opened in Oct4 were closed in the mutant (4293 out of 4367) (Fig 1C-G and EV2C)
Second, ATAC-seq using SKO and SKO-L80A mediated reprogramming showed that the normalized signal intensity in SKO-L80A was lower than in SKO. There was a small subset of genes (1834 out of 14508) that became condensed due to the mutation of Oct4. Further, the ATAC-seq signal intensity at the endogenous Oct4, Sox2 and Nanog loci were much lower in SKO-L80A than in SKO, consistent with the higher methylation levels of H3K9 and H3K27 in the promoter regions of these pluripotency genes in MEFs. Thus, the increased H3K9 and H3K27 methylation levels in SKO-L80A compared with SKO is also consistent with heterochromatin loosening. (Fig 3 and EV3)
Third, as in previous report we indicated that Gadd45a opens up heterochromatin depending on its G39 residue (Chen et al., 2016), we have performed reprograming rescue experiments and showed that Gadd45a could rescue the reprogramming deficiency of SKO-L80A, while its mutant Gadd45a-G39A couldn't (Fig 4D). As Gadd45 is heterochromatin loosener and G39A mutant loses this

function, our rescue data strongly demonstrated Oct4 loosens heterochromatin through its linker, but little chance of some same specific transcriptional responses via both G39 of Gadd45 and L80 of Oct4.

We have added text: "Gadd45a, as well as other family members Gadd45b and Gadd45g, but not its inactive form Gadd45a-G39A, were able to rescue SKO-L80A (Fig 6A and EV4D). As Gadd45a opens up heterochromatin depending on the G39 residue (Chen et al., 2016), our rescue data implicate that the reprogramming deficiency of SKO-L80A should be due to the failure of heterochromatin loosening by Oct4-L80A similar as Gadd45a-G39A."

ii) There is insufficient evidence to support a specific role for Brg1 in this context, versus other chromatin remodellers and complexes. Given these weaknesses, my opinion is that the current manuscript is not supported by firm conclusions, and it lacks sufficient new and specific insight into the role of Oct4 in reprogramming.

Re: We do appreciate this comment, and have designed shRNA of Brg1 to knockdown Brg1 in reprogramming and performed ATAC-seq, and added new data (Fig 4 and EV4A-C), indicating that Oct4 interacts with Brg1 directly in reprogramming depending on its L80 residue. The normalized signal intensity of SKO with Brg1 silencing was lower than that of SKO, but higher than that of SKO-L80A. Most of the gene loci that failed to open (FO) in shBrg1 (90 out of 108) overlapped with FO in Oct4-L80A mutant. The states of specific gene loci such as Klf4 targets Cdh1 and Lefty, epithelial gene Ocln, pluripotency genes Oct4, Sox2 and Nanog were similar in SKO-L80A and SKO plus shBrg1. GO and motif analyses further indicate that knocking down Brg1 could partially mimic the effect of Oct4-L80A on reprogramming

Also, to explore the mechanisms of Oct4 loosening heterochromatin, we have investigated a lot of literature. Many chromatin remodeling proteins such as BAF remodeling complex proteins Brg1 and Baf155 (Singhal et al., 2010), SWI/SNF complex protein INO80 (Wang et al., 2014), and so on, have been reported to be recruited by Oct4 and facilitates reprogramming. Furthermore, Oct4 recruits and co-operates with many epigenetic enzymes such as H3K4 methyltransferase complex component Wdr5, H3K9me2 demethylase Jmjd1c and others to mediate nucleosome depletion and chromatin relaxation (Shakya et al., 2015, Wang et al., 2014, Wu et al., 2015). However, which one is responsible for the deficiency of SKO-L80A, we performed label-free mass spectrometry to compared the interactome of Oct4 and Oct4-L80A and only two proteins exhibited a significantly reduced intensity in the Oct4-L80A interactome: Brg1 and Chd4, a helicase of the NuRD complex (Esch et al., 2013). As Brg1 has been reported to improve reprogramming, while Chd4 has only been studied in mESC maintain, in this manuscript, we selected Brg1 to study.

We have added text: "Several reports have suggested that Oct4 could interact with ATP-dependent chromatin remodeling proteins, such as Brg1, Baf155, INO80 and so on (Ding et al., 2012, Esch et al., 2013, van den Berg et al., 2010). Previously, we compared the interactome of Oct4 and Oct4-L80A and showed two proteins exhibited a significantly reduced intensity in the Oct4-L80A interactome: Brg1 and Chd4 (Esch et al., 2013). As Brg1 has been reported to improve reprogramming (Singhal et al., 2010), we focused on Brg1."

Other major concerns include the absence of key experimental information for several figures, which makes it difficult to interpret the results. For example, Figures 1A and EV1 report FRAP, but of what protein(s)? HP1a and Histone H1? Please also show images of pre-bleach, bleach and recovering nuclei to see the targeted regions. It looks as though the experiments were only performed once. It is also difficult to get a sense of whether the SSEA-1-positive chromatin is 'hyderdynamic' without showing comparative data for MEF and iPSCs.

Re: We do appreciate this comment and have added the description of H1 FRAP. The images of pre-bleach, bleach and recovering nuclei were also added. As shown in Fig EV1B and C, we labeled HP1a with mCherry and histone H1 with GFP. HP1a-mCherry allowed us to distinguish heterochromatin and euchromatin. By selecting Region of Interest within HP1a foci, we performed fluorescence recovery after photobleaching (FRAP) of heterochromatin H1. For each groups, more than 18 cells were analyzed in three independent experiments.

When comparing outcomes between different transcription factors, or between Oct4 and L80A Oct4, it is important to show that protein levels of the expressed factors are comparable.

Re: We do appreciate this comment and have performed western blot to show that the expesssion level of Oct4 and L80A Oct4 were comparable, which was shown in Fig EV1D.

Figure 1B. Are the levels of total HP1a lower in the Oct4 transduced cells compared to the other cells? That is a bit surprising given that HP1a is easily detectable in fully reprogrammed iPSCs.
Re: We do appreciate this comment and we are sorry that we didn't describe it clearly, and have rewirtten. We transduced single factors such as Flag control, Sox2, Klf4, Oct4 and Oct4-L80A into MEFs, and quantified HP1a levels using immunofluorescence. Then we found the level of total HP1a in the Oct4 transduced MEFs were lower than MEFs transduced with Flag, Sox2 or Klf4.

Figure 1C. I can't really tell what I'm looking at here. And there is no attempt to quantify whether the Oct4 loci are inside or outside of chromocentres. Or replicates.
Re: We do appreciate this comment and have quantified the percentage of co-localization between Oct4 loci and HP1a foci (Fig. 1J).

End of page 6: "These results indicated that the Oct4 loosens heterochromatin through its linker, by recruiting BAF complexes, which are known to regulate reprogramming". This conclusion is not supported by the results shown. The data provided shows that Oct4 and Brg1 co-localise (in CHO cells) and interact (when overexpressed in 293 cells), but does not say anything about the regulation of chromatin remodelling in the context of reprogramming or provide a direct role for Oct4 in heterochromatin control.
Re: We do appreciate this comment. Besides using a LacO-LacI targeting system and co-IP assay to show the interaction between Oct4 and Brg1, we have designed shRNA of Brg1 to knockdown Brg1 in reprogramming and performed ATAC-seq, and added new data (Fig 4 and EV4A-C). The normalized signal intensity of SKO with Brg1 silencing was lower than that of SKO, but higher than that of SKO-L80A. Most of the gene loci that failed to open (FO) in shBrg1 (90 out of 108) overlapped with FO in Oct4-L80A mutant. The states of specific gene loci such as Klf4 targets Cdh1 and Lefty, epithelial gene Ocln, pluripotency genes Oct4, Sox2 and Nanog were similar in SKO-L80A and SKO plus shBrg1. GO and motif analyses further indicate that knocking down Brg1 could partially mimic the effect of Oct4-L80A on reprogramming.
We have deleted this sentence and rewritten: "These results indicate that Oct4 interacts with Brg1 through its linker domain."

Figure 2C. Please add IgG control. The inputs, IgG control and IPs should be on same blot. The experiments should ideally be done in SSEA-1 positive reprogramming cells to show that endogenous proteins interact.
Re: We do appreciate this comment and have done the IP experiments in reprogramming cells. And we showed the IgG control, Inputs and IPs on the same blot in the new image. (Fig. 5C)

Figures 3 to 5 need MEF and iPSC samples as references in order to interpret the differences in ChIP levels.
Re: We do appreciate this comment and have added the MEF and mESC samples in the H3K9Me3-ChIP and H3K27Me3-ChIP experiments (Fig 3G and Fig EV4H).

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2nd Editorial Decision 4th Sep 2019

Thank you for re-submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees whose comments are enclosed. As you will see, the referees state that your manuscript has substantially improved during the revision and they are broadly in favour of publication, pending satisfactory minor adjustments.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending the remaining concerns of the referees are addressed in a minor revision by additional analyses or complementary discussion of the findings and introducing caveats where appropriate.

REFEREE REPORTS:

Referee #1:

In this revised manuscript, the authors tried to ask the points raised by the reviewers. To address the functional relationship between the interaction with Brg1, and the rescue effect of vitamin C and Gadd45, they performed ATAC-seq analyses to evaluate these effects and compared these data. As the results, they found that the shRNA-mediated knock-down of Brg1 in OSK reprogramming showed the effect to make the ATAC-seq pattern similar to that of OSK-L80A. Conversely, vitamin C and Gadd45 make the ATAC-seq pattern of OSK-L80A similar to that of OSK. These data strongly support the idea that the impact of L80 mutation is partly due to the loss of interaction with the epigenetic regulators such as Brg1. The addition of these ATAC-seq data indeed improves the quality of this manuscript and now the claim made by the authors is supported by these data. The present manuscript can be considered for publication in EMBO J after revision of the following points.

1. The ATAC-seq revealed that 1834 genes acquire accessible chromatin by OKK but not OSK-L80A (Fig 3B). Among them, 90 genes are shared with the the genes failed to open in OSK+sgBrg1, 50 genes are shared with the opened genes in OSK-L80A+Vc, and 407 genes are shared with the opened genes in OSK-L80A+Gd. How about the overlap between these gene groups (90, 50 and 407)? The overlapping fraction could contain the critical genes for succession of reprogramming.
2. How about the synergy between Vc and Gadd45?
3. Which step of the reprogramming event is enhanced by Vc or Gadd45? Which period of the reprogramming culture required them for improvement of the reprogramming efficiency by OSK L80A?
4. If the effect of Vc and/or Gadd45 is found at the early stage, it should be evaluated by the HP1 FLAP assay as in Fig 1.

Referee #3:

This is a revised and much improved version of the manuscript that I reviewed previously. The authors have performed substantial additional experiments and analyses.

My two primary criticisms at the time were due to the lack of evidence supporting a direct link between a) Oct4 and chromatin remodelling during reprogramming, and b) the role of Brg1 in this context.

My first criticism has been adequately addressed, predominantly by adding ATAC-seq data to provide chromatin accessibility information as a readout of chromatin remodelling. These data have helped demonstrate the chromatin accessibility defects in the Oct4-L80A mutants, to some extent in the Brg1 KD, and restored signal in the rescue experiments.

The second criticism has been partially addressed through Brg1 knockdown experiments. This has

helped to test their model more directly. The effect of Brg1 KD on chromatin accessibility is modest - 95% of the sites affected in the Oct4 L80A mutants are not affected by Brg1 KD. And although chromatin is less accessible at several Klf4 target sites after Brg1 KD, the authors do not show if this alters Klf4 binding to these regions, as would be predicted from their model. Nevertheless, I think that overall there is enough evidence to support a function for Oct4 in opening up chromatin to enable Klf4 binding and activation of target genes. Given the current interest in cooperative interactions between reprogramming factors then the manuscript makes a valuable contribution towards this area.

Minor
There seems to be some text missing in the abstract, fourth line.

2nd Revision - authors' response 5th Sep 2019

Referee #1:

In this revised manuscript, the authors tried to ask the points raised by the reviewers. To address the functional relationship between the interaction with Brg1, and the rescue effect of vitamin C and Gadd45, they performed ATAC-seq analyses to evaluate these effects and compared these data. As the results, they found that the shRNA-mediated knock-down of Brg1 in OSK reprogramming showed the effect to make the ATAC-seq pattern similar to that of OSK-L80A. Conversely, vitamin C and Gadd45 make the ATAC-seq pattern of OSK-L80A similar to that of OSK. These data strongly support the idea that the impact of L80 mutation is partly due to the loss of interaction with the epigenetic regulators such as Brg1. The addition of these ATAC-seq data indeed improves the quality of this manuscript and now the claim made by the authors is supported by these data. The present manuscript can be considered for publication in EMBO J after revision of the following points.
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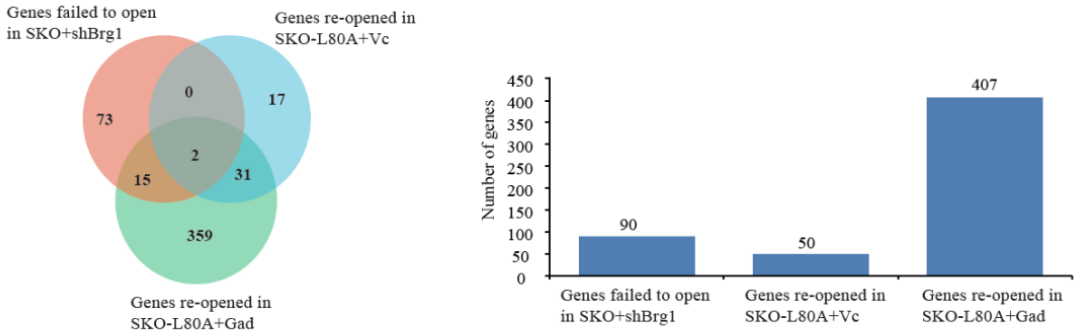
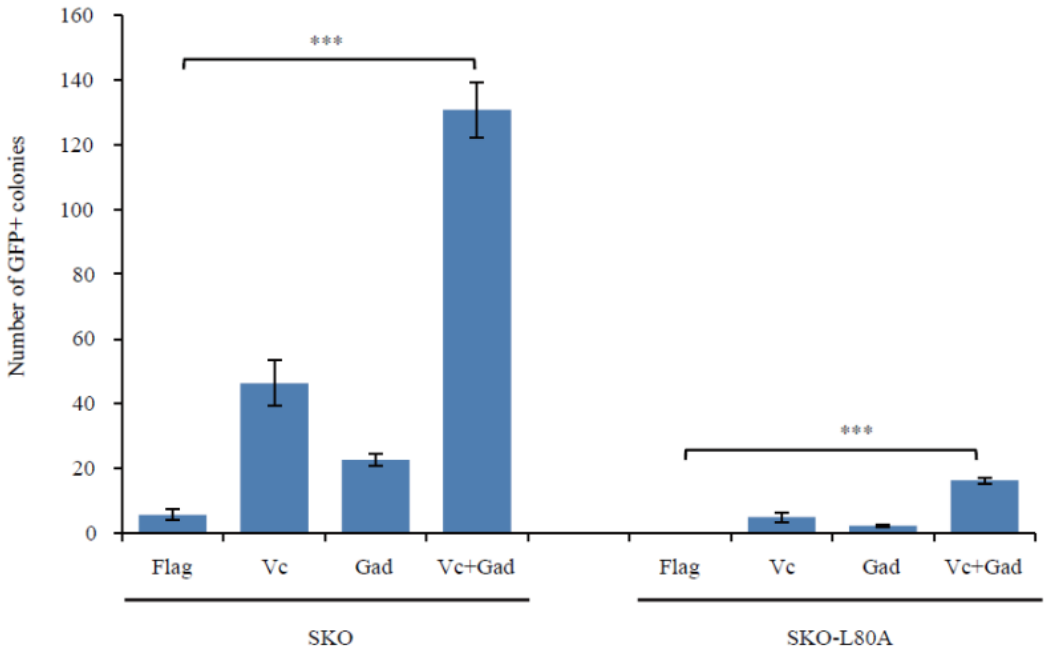
Re: we do appreciate this comment, and have performed analyses to show the overlap between these gene groups. As shown in the following figure, there were two genes identified in the overlapping fraction, Dpp4 and Grtp1.
Dipeptidyl-peptidase 4 (Dpp4) is an exopeptidase which selectively cleaves N-terminal dipeptides from substrates including cytokines, growth factors, neuropeptides and hormones. The dysregulation of Dpp4 is related to many diseases, such as inflammation, cancer, obesity, and diabetes. (Rohrborn et al., 2015) Recently, Dpp4 was identified as one of the prominently upregulated secreted factors during reprogramming (Bansho et al., 2017). LIF, BMP4 and FGF2 are cytokines important for pluripotency regulation and have a putative truncation site for Dpp4 (Ou et al., 2013), implicating Dpp4 may play important role in pluripotency maintain or in reprogramming.
GH regulated TBC protein-1 (Grtp1) is highly expressed in testes and regulated by growth hormone. The expression level of Grtp1 is down regulated in Rex1 knockout mice (Lu et al., 2001, Rezende et al., 2011).



2. How about the synergy between Vc and Gadd45?

Re: we do appreciate this comment, and have performed the experiments. As shown in following figure, when Gadd45a was overexpressed in the presence of Vc, we got more iPS colonies in both SKO and SKO-L80A mediated reprogramming. These results suggest that Vc and Gadd45a enhance somatic cell reprogramming in different ways.



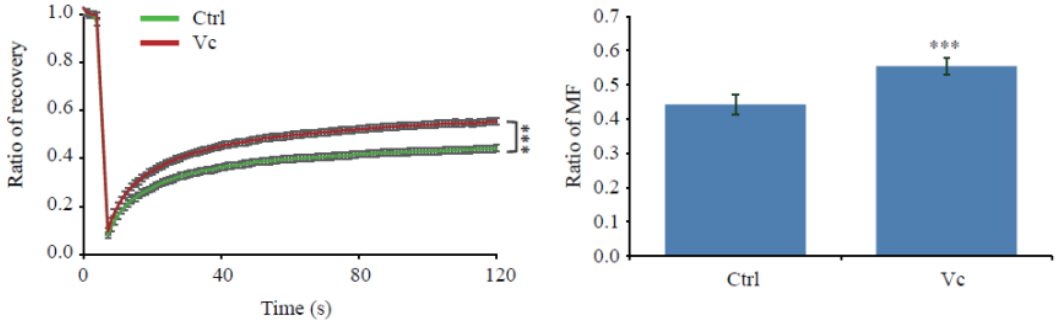
3. Which step of the reprogramming event is enhanced by Vc or Gadd45? Which period of the reprogramming culture required them for improvement of the reprogramming efficiency by OSK L80A?

Re: we do appreciate this comment and have added discussion in the text:" Thus, our results demonstrated Vc or Gadd45a takes part in the MET by enhancing Klf4's binding in the early stage of reprogramming and complements Oct4-L80A's heterochromatin loosening defects to rescue reprogramming efficiency.".

According to our previous reports and data in this work, we summarized that Vc functions during the whole process of reprogramming. At the early stage, Vc could increase the chromatin accessibility and decrease the histone modification H3K36Me2/3 (Esteban et al., 2010, Wang et al., 2011). Vc could also modulate TET1 function at loci critical for MET in reprogramming (Chen et al., 2013a). At the late stage, Vc could convert pre-iPSCs into iPSCs by enhancing H3K9Me3 demethylation (Chen et al., 2013b, Esteban et al., 2010).

Gadd45a functions in the early and middle stage of reprograming (Chen et al., 2016). It could relax the heterochromatin and increase the chromatin accessibility.
In SKO-L80A reprogramming, they may function in a similar pattern as in SKO reprogramming. In this work, we found that they also take part in the MET by enhancing Klf4's binding in the early stage.

4. If the effect of Vc and/or Gadd45 is found at the early stage, it should be evaluated by the HP1 FLAP assay as in Fig 1.
Re: we do appreciate this comment and have performed experiments. In our previous report, we have showed that Gadd45a increased the heterochromatin H1 dynamics by H1-FRAP and decreased the HP1a level by HP1a staining (Chen et al., 2016). As shown in the following figure, we performed H1-FRAP and found that Vc could also increase the heterochromatin H1 dynamics.



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Minor
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Re: we do appreciate this comment and have corrected the mistake.
References
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3rd Editorial Decision 6th Sep 2019

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Duanqing Pei and Xingguo Liu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99165R-Q

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Please refer to the legends for Figure 1A, B and E (page36-37), Figure 5B (page 40) and Figure EV5 (page 46).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, please refer to Materials and methods-Statistics (page 29).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA

Is there an estimate of variation within each group of data?	Yes, please refer to Materials and methods-Statistics (page 29).
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Please refer to Materials and methods-Immunofluorescence (page 24), Immuno-FISH (page 24), Western blot (page 25), Co-immunoprecicpitation (page 25), ChIP (page 26).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the cells used (including Plat-E, mESCs and primary fibroblasts) were described in Materials and methods-DNA constructs, cell lines, and cell culture (page 21), and were routinely checked for mycoplasma contamination (Lonza, MycoAlert kit) and they were all negative.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The human skin fibroblast were obtained with approval from the ethics committee of the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. Please refer to Materials and methods-DNA constructs, cell lines, and cell culture (page 21)
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Please refer to Materials and methods-Accession numbers (page 29).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA